Transcriptional coactivator PGC-1α regulates chondrogenesis via association with Sox9

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Chondrogenesis is a multistep pathway in which multipotential mesenchymal stem cells (MSC) differentiate into chondrocytes. The transcription factor Sox9 (SRY-related high mobility group-Box gene 9) regulates chondrocyte differentiation and cartilage-specific expression of genes, such as *Col2a1* (collagen type II α1). However, *Sox9* expression is detected not only in chondrogenic tissue but also in nonchondrogenic tissues, suggesting the existence of a molecular partner(s) required for Sox9 to control chondrogenesis and chondrogenic gene expression. Here, we report identification of peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1α) as a coactivator for Sox9 during chondrogenesis. Expression of *PGC-1α* is induced at chondrogenesis sites during mouse embryonic limb development and during chondrogenesis in human MSC cultures. PGC-1α directly interacts with Sox9 and promotes Sox9-dependent transcriptional activity, suggesting that PGC-1α acts as a transcriptional coactivator for Sox9. Consistent with this finding, PGC-1α disruption in MSC by small interfering RNA inhibits *Col2a1* expression during chondrogenesis. Furthermore, overexpression of both *PGC-1α* and *Sox9* induced expression of chondrogenic genes, including *Col2a1*, followed by chondrogenesis in the MSC and developing chick limb. Together, our results suggest a transcriptional mechanism for chondrogenesis that is coordinated by PGC-1α.

cartilage | mesenchymal stem cell | peroxisome proliferator-activated receptor γ | coactivator 1α | limb development

Chondrogenesis is a tightly regulated process in which multipotential mesenchymal stem cells (MSC) differentiate into chondrocytes to form cartilage (1, 2). This process is initiated by commitment to the chondrogenic lineage and condensation of MSC, followed by differentiation into chondrocytes that is associated with expression of cartilage-specific genes. These genes include components of cartilage extracellular matrix genes, such as those encoding collagen type II α1 (*Col2a1*), type IX collagen, *aggrecan*, link protein, and cartilage oligomeric matrix protein (*COMP*), at various kinetics of induction. Expression of these genes is regulated at the transcriptional level (3), spatially and temporally, so that they have different and dynamic expression patterns during chondrogenic differentiation (4, 5). Subsequently, chondrocytes proliferate and secrete a cartilage-specific matrix to form the cartilage anlagen.

The transcription factor Sox9 (SRY-related high mobility group-Box gene 9) is a key regulator of chondrogenic differentiation and chondrogenic gene expression (6). For example, mice lacking *Sox9* function display distortion of numerous cartilage-derived skeletal structures (7). Despite its importance for chondrogenesis, the mechanisms by which Sox9 regulates cartilage-specific transcription are poorly understood. For instance, expression of *Sox9* is detected in nonchondrogenic tissues, such as the genital ridge (6), but it activates chondrocyte-specific genes only in the chondrocyte cell lineage. We recently revealed that Sox9 activates *Col2a1* via interaction with cAMP response element-binding protein binding protein (CBP)/p300, a histone acetyl transferase (8). However, CBP/p300 is expressed almost ubiquitously, and, thus, the mechanisms for cartilage-specific transcriptional regulation is yet to be elucidated. These facts strongly suggest that an additional factor is required for Sox9 to activate expression of its target genes in a tissue-specific manner.

Insight into the molecule that might cooperate with Sox9 for chondrogenesis came from our whole-mount *in situ* hybridization analysis of mouse embryos with genes encoding transcriptional cofactors. In this experiment, we found that *PGC-1α*, which is known to play a role in adaptive thermogenesis and gluconeogenesis as a cofactor for a nuclear receptor, peroxisome proliferator-activated receptor γ (9, 10), is expressed at chondrogenic sites. This fact prompted us to investigate the role of peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1α) in chondrogenesis. In this article, we show that PGC-1α acts as a coactivator for Sox9 to regulate chondrogenesis.

Materials and Methods

Chondrogenesis of MSC. Human MSC were purchased from Bio-Whittaker. To induce chondrogenesis, 2.5×10^5 cells were treated in pellet culture with chondrogenic induction medium (11) with TGF-β3 (10 ng/ml) as described (8) up to 2 weeks. Infection of adenovirus was carried out by incubating dissociated MSC with adenovirus for 1 h at 37°C, followed by formation of a pellet culture. Adenovirus was made with the AdEASY system following the manufacturer's instructions (Stratagene; also in ref. 12, in which transgene expression was driven by the CMV promoter). Purification and concentration of recombinant adenoviruses were done as described (13).

Tissue Culture and Transfections. Mature chondrocytes were isolated from human cartilage, and a primary cell culture was established (14). After passaging twice, cells were cultured at high density with alginate beads for 7 days (15). The human chondrosarcoma cell line SW1353 was grown in the same conditions described above. Cells were transfected by using Fugene 6 (Roche Diagnostics) as described (8). Comparable expression levels of Gal-Sox9 and PGC-1α polypeptides were verified by Western blotting.

***In Vitro* Analyses.** For immunoprecipitation assay, cells were washed and resuspended in RIPA buffer (0.15 mM NaCl/0.05 mM Tris·HCl, pH 7.2/1% Triton X-100/1% sodium deoxy-

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Abbreviations: PGC-1α, peroxisome proliferator-activated receptor γ coactivator 1α; Sox9, SRY-related high mobility group-Box gene 9; MSC, mesenchymal stem cells; Col2a1, collagen type II α1; siRNA, small interfering RNA; RCAS, replication-competent avian retrovirus; E*n*, embryonic day *n*.

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cholate/0.1% SDS) and treated as described (8). Immunoprecipitations were performed with 5 μl of polyclonal anti-PGC-1α antibody (SC5815, Santa Cruz Biotechnology) or monoclonal antihemagglutinin (Y11, Santa Cruz Biotechnology). Western blotting was performed by using polyclonal anti-Sox9 (AB5809, Chemicon) or anti-FLAG (M2, Sigma). The GST pull-down and chromatin-immunoprecipitation assays were carried out as described (8) (see *Supporting Text*, which is published as supporting information on the PNAS web site).

Small Interfering RNA (siRNA). The sequences of double-stranded nucleotides used for siRNA (Dharmacon, Lafayette, CO) are shown in *Supporting Text*. Cells cultured in a six-well plate (2×10^5 per well) were transfected with 150 μmol of siRNA with OligofectAMINE (Invitrogene), and 24 h after transfection, cells were cultured in pellets for the indicated days.

Real-Time PCR. Total RNA was extracted and oligo(dT)-primed cDNA was prepared from 500 ng of total RNA by using Superscript II (Invitrogen). The resulting cDNAs were analyzed by using the Taqman system for quantitative analysis of specific transcripts according to the manufacturer's instructions (Applied Biosystems). All mRNA expression data were normalized to *GAPDH* expression in the corresponding sample (16). The sequences of TaqMan probes used are described in *Supporting Text*.

***In Situ* Hybridization.** Whole-mount *in situ* hybridization on mouse and chick embryos was carried out according to the standard procedure (17). The mouse *PGC-1α* probe contains 2.4 kb of the coding sequence. Other probes have been described (4, 6, 7).

Injection of Adenovirus and Retrovirus into Developing Chick Limb Buds. Overexpression of *PGC-1α* and/or *Sox9* in developing chick limb buds is accomplished by using viral vectors following standard injection procedures (13, 17). In brief, adenovirus–*PGC-1α* was infected into the limb bud mesenchyme of Hamburger–Hamilton (HH) stage 19–21 embryos (18). Replication-competent avian retrovirus (RCAS)-carrying *Sox9* was infected into the lateral plate mesoderm of HH stage 9–11 embryos, which will give rise to the limb bud mesenchyme. The difference in injection timing is caused by the characters of each virus: adenovirus expresses the transgene within 1 day after infection, whereas RCAS requires integration into the host genome and takes 1–2 days to express the transgene at high levels in this experimental system. The embryos were allowed to develop further and fixed at the desired stage for further analyses (17).

Results

Expression of PGC-1α Correlates with Chondrogenesis. In addition to transcription factors, it is known that transcriptional coactivators are involved in gene expression and thus regulate various biological programs (19). To gain insights into the function of coactivators in chondrogenesis, we analyzed the expression of various coactivators during mouse embryogenesis. In the course of this study, we unexpectedly found that *PGC-1α* is expressed dominantly in the chondrogenic region in the developing mouse limb bud (Fig. 1 and Fig. 5, which is published as supporting information on the PNAS web site). The signal was associated with digit primordia in both forelimbs and hindlimbs. PGC-1α originally was discovered as a peroxisome proliferator-activated receptor γ coactivator, and it has been demonstrated that PGC-1α regulates adipocyte differentiation (10), gluconeogenesis (20, 21), and mitochondrial respiration (22). During these biological events in adult tissues, the expression of *PGC-1α* changes dramatically as it regulates the activity of targeted nuclear factors. However, its role in embryonic development has not been characterized. To examine the possible involvement of *PGC-1α* in chondrogenesis, we further analyzed its expression

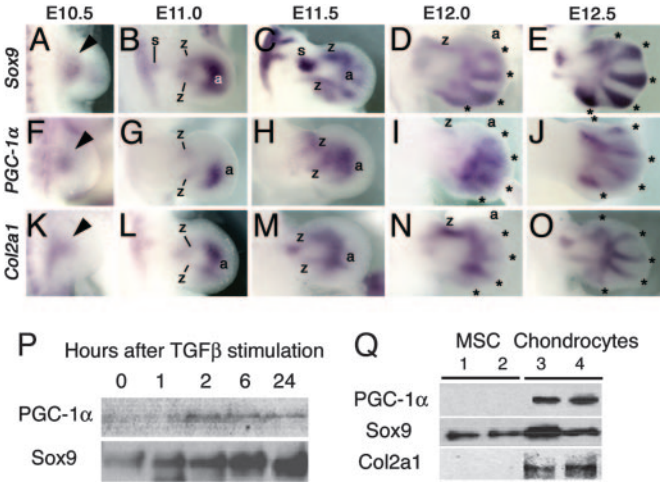

Fig. 1. Expression of *PGC-1α* correlates with chondrogenesis. (*A–O*) Comparison of expression patterns of *Sox9* (*A–E*), *PGC-1α* (*F–J*), and *Col2a1* (*K–O*) in developing mouse forelimb buds at indicated embryonic stages. The presumptive stylopod (s) and zeugopod (z) elements are indicated. The autopod area (a) is also indicated. In *D*, *I*, and *N*, only autopod and part of zeugopod areas are shown, and in *E*, *J*, and *O*, only the autopod area is shown because the expression in the proximal region becomes faint. Arrowheads mark expression in the core region at E10.5. Asterisks mark tips of the digit primordia. Shown are dorsal views with the anterior to the top and the distal to the right. (*P*) Comparison of the time course of PGC-1α and Sox9 protein level during chondrogenesis of MSC. After being stimulated by TGF-β, whole-cell lysates of MSC were prepared at the indicated time points. Protein levels of PGC-1α and Sox9 were analyzed by Western blotting. (*Q*) The protein levels of PGC-1α, Sox9, and Col2a1 were compared between undifferentiated human MSC (lanes 1 and 2) and mature chondrocytes from two independent donors (lanes 3 and 4) by Western blotting.

pattern and compared it with that of *Sox9* and *Col2a1*, important genes in chondrogenic differentiation *in vivo*. Expression of *PGC-1α* is detected in the core region of the developing limb at embryonic day 10.5 (E10.5) in a pattern similar to *Col2a1* (Fig. 1 *F* and *K*). *Sox9* is also expressed at the core region (Fig. 1*A*). As the limb grows, the expression of *PGC-1α* exhibits a dynamic pattern, and it extends toward zeugopod and autopod regions (E11.0–11.5, Fig. 1 *G* and *H*). Such a dynamic expression pattern is also observed with *Sox9* in a wider region of the mesenchyme, consistent with *Sox9* being required for the initial condensation process (Fig. 1 *A–C*). *Col2a1* gene expression, on the other hand, is more restricted as compared with that of *Sox9*, is similar to that of *PGC-1α*, and changes over time from being expressed in the proximal core region to being distally expressed (Fig. 1 *K–O*). These expression patterns start to fade from the proximal region and begin to be strongly detected in the autopod region at E12.0–12.5 (Fig. 1 *D*, *E*, *I*, *J*, *N*, and *O*). In this region, *PGC-1α* marks the digital primordia, and *Sox9* is detected in a broader region than *PGC-1α* and *Col2a1*. Overall, the spatiotemporal pattern of *PGC-1α* expression correlates with that of *Sox9* and *Col2a1* and supports the idea that PGC-1α may have an important role in chondrogenesis.

We also examined *PGC-1α* expression in a defined *in vitro* system, in which human MSC from bone marrow differentiate into chondrocytes (5, 11). To induce chondrogenic differentiation, MSC were transferred to micromass cultures with TGF-β, a factor previously shown to induce chondrogenesis in MSC (5, 11). Consistent with the observations in mouse limb buds, we observed up-regulation of both PGC-1α and Sox9 after TGF-β stimulation (Fig. 1*P*). We found evident induction of PGC-1α at 2 h poststimulation, and it was maintained until 24 h poststimulation. Sox9 expression was detected in the unstimulated MSC,

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and it was up-regulated at 1 h poststimulation and increased until 24 h poststimulation. To further examine the correlation between *PGC-1α* expression and chondrogenesis, we compared undifferentiated MSC and mature chondrocytes from human cartilage, by monitoring the levels of PGC-1α, Sox9, and Col2a1 (Fig. 1*Q*). PGC-1α as well as Sox9 and Col2a1 were detected in mature chondrocytes from two different donors. In MSC, on the other hand, we did not detect PGC-1α and Col2a1, whereas Sox9 was detected. These results suggest that *PGC-1α* expression correlates with chondrogenesis in MSC.

PGC-1α Regulates Sox9-Dependent Transcriptional Activity on *Col2a1* Enhancer. The above results lead us to hypothesize that PGC-1α might act as a coactivator for Sox9 during chondrogenesis. To examine this idea, we used a variety of *in vitro* analyses with the *Col2a1* gene as a target of Sox9–PGC-1α activity. *Col2a1* is a well characterized chondrogenic gene, and its expression is induced during early stages of chondrocyte differentiation in a Sox9-dependent manner at the transcriptional level (23, 24).

First, we investigated the functional significance of PGC-1α on Sox9-dependent reporter assays, using a luciferase reporter plasmid containing part of the *Col2a1* enhancer containing the Sox9 binding site (pKN185luc) (3). We observed significant up-regulation of reporter activity (Fig. 2*A*) in the SW1353 cell line, which expresses *Sox9* (data not shown), demonstrating that *PGC-1α* enhances Sox9-dependent reporter activity. To further verify the action of PGC-1α on Sox9-mediated transactivation, we used the Gal4 fusion system with *Sox9* and a Gal4–Luc reporter containing 5-Gal4 binding sites and the thymidine kinase promoter. Transfection of *Gal4–Sox9* increased luciferase activity ≈1.5-fold as compared with the basal level (Fig. 2*B*). Importantly, transfection of *PGC-1α* in conjunction with *Gal4–Sox9* increased luciferase activity ≈5.5-fold compared with *Gal4–Sox9* (Fig. 2*B*). These data suggest that PGC-1α functionally contributes to Sox9-dependent *Col2a1* gene expression.

Second, we performed an endogenous chromatin immunoprecipitation assay with human chondrocytes (Fig. 2*C*). We clearly observed that PGC-1α and Sox9 are precipitated with a *Col2a1* enhancer fragment, where Sox9 is known to bind and activate expression of the *Col2a1* gene. These results indicate that PGC-1α forms a complex with Sox9 on the *Col2a1* gene.

Third, we examined the physical interaction between PGC-1α and Sox9 by coimmunoprecipitation assay (Fig. 2*D*). We used human chondrocytes that express endogenous PGC-1α and Sox9 to address their endogenous interaction (Fig. 1*Q*). The precipitation was done with anti-PGC-1α antibody, followed by Western blotting with anti-Sox9 antibody. The result demonstrates an endogenous molecular interaction between Sox9 and PGC-1α.

Lastly, we performed GST pull-down assays to examine the direct interaction between PGC-1α and Sox9 (Fig. 2*E*). By using GST-Sox9 protein and *in vitro*-translated PGC-1α protein, we observed binding of PGC-1α (amino acids 6–797; Fig. 2*Ei*) with Sox9, suggesting a direct interaction. To define the domain within PGC-1α, we created a variety of constructs (Fig. 2*E*). The PGC-1α protein contains two LXXLL domains at amino acids 142 and 209, previously shown to be required for an interaction with a transcription factor, hepatocyte nuclear factor (HNF) 4α (21). We observed an interaction between PGC-1α (amino acids 6–180; Fig. 2*Eii*) and Sox9, whereas PGC-1α (amino acids 180–797; Fig. 2*Eviii*) did not interact with Sox9, suggesting that LXXLL (amino acid 209) does not contribute to the interaction with Sox9. We also did not observe an interaction between Sox9 and PGC-1α (amino acids 406–797; Fig. 2*Eix*), which contains the RNA processing motif. To further characterize the interaction domain within PGC-1α (amino acids 6–180; Fig. 2*Eii*), we created PGC-1α (amino acids 6–90; Fig. 2*Eiv*) lacking the LXXLL domain (amino acid 142) and PGC-1α (amino acids 90–180; Fig. 2*Evii*) that is complementary to PGC-1α (amino acids 6–90; Fig. 2*Eiv*). We also made PGC-1α [amino acids 6–180 (L145A); Fig. 2*Eiii*], in which we introduced a point mutation to L145. This point mutation is known to disturb the interaction with HNF-4α (21), and we observed that the mutation abolished the interaction with GST–HNF-4α in the GST pull-down assay (data not shown). Whereas PGC-1α (amino acids 90–180; Fig. 2*Evii*) did not interact with Sox9, we observed the interaction of Sox9 with both PGC-1α (amino acids 6–90; Fig. 2*Eiv*) and PGC-1α [amino acids 6–180 (L145A); Fig. 2*Eiii*]. This result suggests that the interaction between PGC-1α (amino acids 6–180; Fig. 2*Eii*) and Sox9 does not require the LXXLL (amino acid 142) domain. We further characterized the Leu-rich domain in PGC-1α (amino acids 6–90; Fig. 2*Eiv*) by mutating all of the Leu residues in the Leu-rich domain [amino acids 6–90

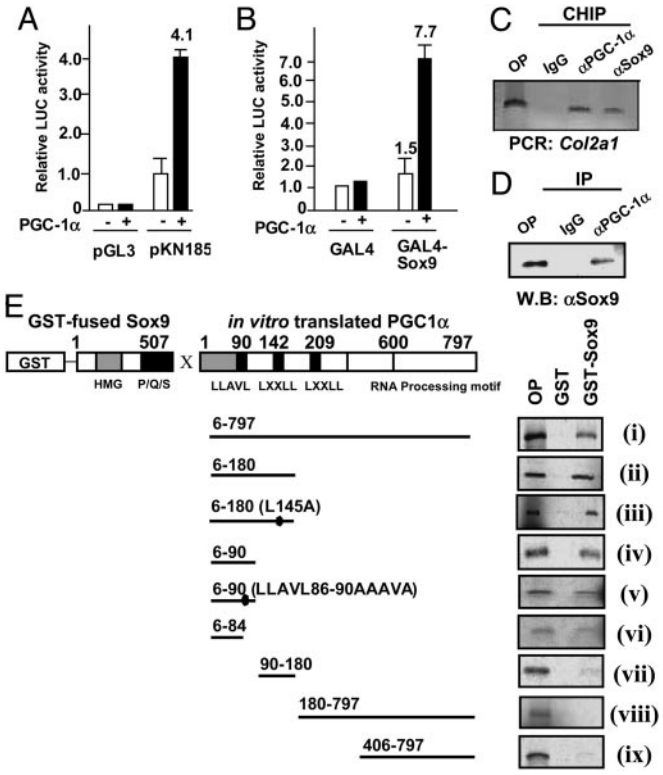

Fig. 2. PGC-1α regulates Sox9-dependent *Col2a1* gene expression by direct association with Sox9. (*A* and *B*) PGC-1α potentiates transcriptional activity of Sox9 in a luciferase (LUC) reporter assay. (*A*) SW1353 cells were transfected with reporter plasmids of pGL3-control or pKN185luc containing the *Col2a1* enhancer sequence. Overexpression of *PGC-1α* enhanced reporter activity of pKN185, which is regulated by endogenous Sox9, but not that of the pGL3 control vector. (*B*) Reporter plasmid containing Gal4 binding sites was transfected into SW1353 cells with either Gal4 or Gal4–Sox9. Cotransfection of *PGC-1α* enhanced Gal4–Sox9 activity. (*C*) PGC-1α forms a complex with Sox9 on the *Col2a1* enhancer. Nuclear extract of human chondrocytes was subjected to chromatin immunoprecipitation (CHIP) assay either with anti-PGC-1α or anti-Sox9 antibody. Immunoprecipitated *Col2a1* enhancer was detected by PCR. OP, onput. (*D*) Endogenous interaction between Sox9 and PGC-1α in human chondrocytes by coimmunoprecipitation (IP). Lysates of human chondrocytes were immunoprecipitated with anti-PGC-1α antibody. Precipitates and 10% onputs (OP) were subjected to Western blotting (W.B.) with anti-Sox9 antibody. (*E*) Mapping of the Sox9-interaction domain within PGC-1α by GST pull-down assay. Deletion mutants of *in vitro*-translated PGC-1α were incubated with either GST or a GST-fused Sox9, and pull-down assay was performed to examine specific interactions. The constructs contain amino acids 6–797 (*i*), 6–180 (*ii*), 6–180 (L145A) (*iii*), 6–90 (*iv*), 6–90 (LLAVL86–90 AAAVA) (*v*), 6–84 (*vi*), 90–180 (*vii*), 180–797 (*viii*), and 406–797 (*ix*) of mouse PGC-1α. Results with each deletion mutant are shown (*Right*). HMG, high mobility group; OP, onput.

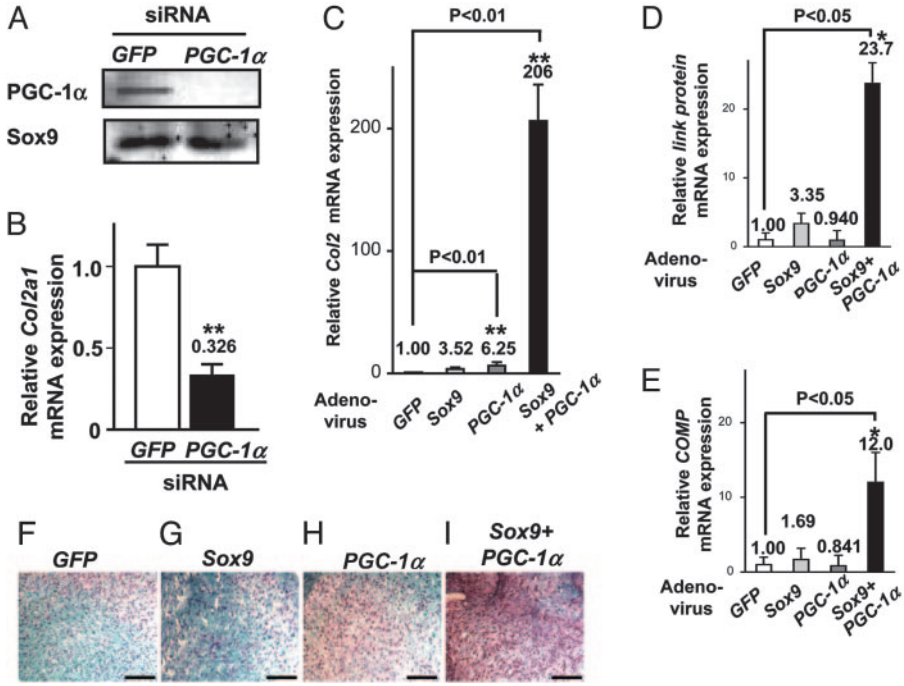

Fig. 3. Sox9 and PGC-1α regulates chondrogenesis *in vitro*. (*A*) Down-regulation of PGC-1α by siRNA in MSC. MSC were transfected by oligo-siRNA for *GFP* (*Left*) or *PGC-1α* (*Right*) and cultured, followed by measurement of protein levels of PGC-1α and Sox9 by Western blotting. PGC-1α–siRNA specifically and efficiently down-regulated protein levels of PGC-1α. (*B*) PGC-1α is essential for *Col2a1* expression during chondrogenesis. Expression level of *Col2a1* was measured by real-time PCR in the same experiment as in *A*. (*C–E*) Sox9 and PGC-1α synergistically activates expression of chondrogenic maker genes in MSC during chondrogenesis. Human MSC were infected with adenovirus and subjected to pellet culture for chondrogenesis. The expression levels of *Col2a1* (*C*), the gene encoding link protein (*D*), and *COMP* (*E*) were analyzed by real-time PCR. In *B–E*, results are shown by mean ± SD, and the significance was examined by Student's *t* test. (*F–I*) Safranin-O staining of the cartilage matrix of MSC. MSC were infected with the indicated adenoviruses, pellet-cultured, and stained with safranin-O after sectioning (8 μm). (Bars: 100 μm.)

(LLAVL86–90AAAVA); Fig. 2*Ev*] and deleting this domain (amino acids 6–84; Fig. 2*Evi*), and they did not significantly affect the interaction with GST–Sox9. This detailed characterization resulted in the identification of a unique domain, the amino-terminal region, as the Sox9 interaction domain. A complementary experiment revealed that Sox9 (amino acids 328–507), containing a transactivation domain (23), bound PGC-1α (Fig. 6, which is published as supporting information on the PNAS web site).

PGC-1α Regulates Sox9-Dependent Chondrogenesis in MSC and Chondrocytes. To examine the role of PGC-1α, we used chemically synthesized siRNA against *PGC-1α* in conjunction with TGF-β stimulation for chondrogenesis in MSC. Transfection of siRNA against *PGC-1α* resulted in down-regulation of PGC-1α protein levels (Fig. 3*A*) that is normally induced during chondrogenesis (Fig. 1*P*), whereas PGC-1α–siRNA did not affect the level of Sox9 protein. PGC-1α–siRNA also down-regulated *Col2a1* expression in MSC during TGF-β-stimulated chondrogenesis with a statistical significance (Fig. 3*B*). Although there was still detectable *Col2a1* expression, these data demonstrate that PGC-1α is an important factor for *Col2a1* expression during chondrogenesis.

To address the role of *PGC-1α* in Sox9-dependent chondrogenesis, we overexpressed *PGC-1α* and *Sox9* by using adenovirus, alone or in combination, in MSC, followed by gene expression analysis by quantitative real-time PCR (Fig. 3 *C–E*). *Col2a1* gene expression (Fig. 3*C*) was up-regulated by *Sox9* alone (3.52-fold), as compared with the control, adenovirus–*GFP*. It is also up-regulated by *PGC-1α* alone (6.25-fold) with a statistical significance. This finding is consistent with the result that overexpressing *PGC-1α* in mature chondrocytes led to an increase of the Col2a1 protein level (Fig. 7, which is published as supporting information on the PNAS web site). Importantly, double infection of both *Sox9* and *PGC-1α* resulted in a significant increase of *Col2a1* levels (206-fold), demonstrating a synergistic action between *Sox9* and *PGC-1α* on the expression of *Col2a1* during chondrogenesis. We also examined the transcript level of other chondrogenic genes, including those encoding link protein (Fig. 3*D*) and *COMP* (Fig. 3*E*). Although *PGC-1α* alone could not activate these genes as much as *Col2a1*, the divergence of the up-regulation might be caused by differences in their dependence on PGC-1α activity. In agreement with this idea, we observed slight, but not significant, down-regulation of the expression of the gene encoding link protein and *COMP* by the knockdown of *PGC-1α* with siRNA (Fig. 8, which is published as supporting information on the PNAS web site). Importantly, in the MSC doubly infected with Sox9 and PGC-1α, we observed significant up-regulation in the expression of these genes (Fig. 3 *D* and *E*). On the other hand, we did not observe such synergistic activation in the mRNA level of both mitochondrial transcription factor A and ATP synthetase (whose genes are known to be regulated by PGC-1α) in the same samples (data not shown), confirming the specific role of PGC-1α during Sox9-dependent chondrogenesis. The up-regulation of the transcript levels of the gene encoding link protein and *COMP*, in addition to that of *Col2a1*, suggests that the chondrogenesis process has been significantly provoked by double infection of *Sox9* and *PGC-1α*.

We also examined cartilage matrix accumulation in MSC infected by the adenoviruses by using safranin-O staining, in which the cartilage matrix is stained red. Infection of MSC with *Sox9* did not show significant differences (Fig. 3*G*) compared with the control (Fig. 3*F*). Infection of *PGC-1α* resulted in an increase of cartilage matrix accumulation (Fig. 3*H*). Furthermore, double infection of *Sox9* and *PGC-1α* resulted in significant increase of cartilage matrix accumulation shown in a deep

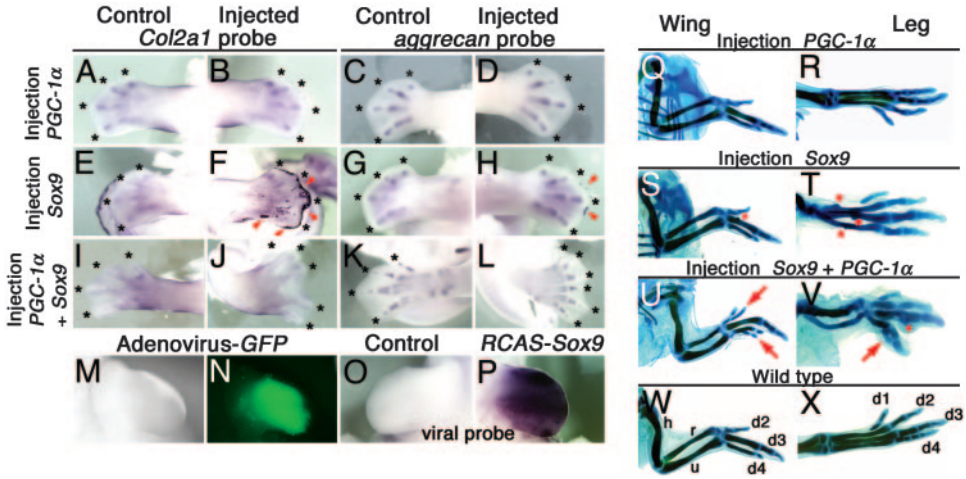

Fig. 4. Sox9 and PGC-1α cooperate in chondrogenesis *in vivo*. (*A–P*) Gene expression analysis of chick limb buds overexpressed with *PGC-1*α (*A–D*), *Sox9* (*E–H*, *O*, and *P*), both *Sox9* and *PGC-1*α (*I–L*) or *GFP* (*M* and *N*). Expression patterns of *Col2a1* (*A*, *B*, *E*, *F*, *I*, and *J*) and the gene encoding aggrecan (*C*, *D*, *G*, *H*, *K*, and *L*) were analyzed. In control experiments, the transgene product, GFP (*M* and *N*) or the viral message (*O* and *P*) was monitored. In *A–P*, the right limb buds received the viral injections (*B*, *D*, *F*, *H*, *J*, *L*, *M*, *N*, and *P*), and the left limb buds of the same embryos served as a control (*A*, *C*, *E*, *G*, *I*, *K*, and *O*). The asterisks indicate the distal tip of the digit primordia. All images are dorsal views. To evaluate virus systems, the bright-field image (*M*) and the dark-field image (*N*) of the same limb bud 20 h after injection of adenovirus-*GFP* are shown, and the RCAS infection was detected throughout limb bud mesenchyme at Hamburger–Hamilton (HH) stage 23 (*O* and *P*). (*Q–X*) Skeletal preparation of chondrogenic matrix stained by Alcian blue at HH stage 36. Chick embryonic wing (*Q*, *S*, *U*, and *W*) and leg (*R*, *T*, *V*, and *X*) after overexpression of *PGC-1*α (*Q* and *R*), *Sox9* (*S* and *T*), or both *Sox9* and *PGC-1*α (*U* and *V*) are shown. The WT (*W* and *X*) is shown for comparison. The skeletal elements are indicated only in WT (*W* and *X*) for simplicity. h, humerus; r, radius; u, ulna; d1, digit I; d2, digit II; d3, digit III; d4, digit IV.

red color (Fig. 3*I*). These results are consistent with our gene expression analysis in MSC overexpressed with *Sox9* and/or *PGC-1*α and further support our theory that PGC-1α acts as a coactivator for Sox9 during chondrogenesis.

PGC-1α and Sox9 Cooperate in Chondrogenesis *in Vivo*. To further examine the role of PGC-1α during chondrogenesis, we made use of developing chick limb buds. This system allows us to analyze the function of genes *in vivo* by viral-mediated overexpression (13, 25). We focused on an analysis of ectopic induction of target genes and chondrogenesis by using *Col2a1* and the gene encoding aggrecan, because their transcripts have been shown to be highly correlated with chondrogenesis in developing chick limb buds (4).

We did not observe any significant change in the expression pattern of *Col2a1* and the gene encoding aggrecan with overexpression of *PGC-1*α (Fig. 4 *A–D*, $n = 42$ and $n = 26$ for *Col2a1* and *aggrecan*, respectively). Both *Col2a1* and aggrecan expression was observed in digital primordia (marked by * in Fig. 4) without ectopic expression. Correlating with this finding, we did not observe ectopic cartilage by overexpressing *PGC-1*α at later stages (Fig. 4 *Q* and *R*, $n = 20/20$), as compared with the WT (Fig. 4 *W* and *X*), although a transient, accelerated cartilage matrix accumulation was observed ($n = 16/75$, data not shown). We used adenovirus–*GFP* to examine tissue integrity and transgene expression and confirmed that the adenovirus system effectively overexpresses the transgene in developing limb buds (Fig. 4 *M* and *N*, $n = 15/15$).

Overexpression of *Sox9* via RCAS virus induced ectopic expression of *Col2a1* and the gene encoding aggrecan. This up-regulation was detected in a scattered manner, predominantly in the distal ridge of the limb (Fig. 4 *E–H*, $n = 18/44$ and $n = 4/28$ for *Col2a1* and *aggrecan*, respectively). We examined expression of viral message by *in situ* hybridization for a viral gene and confirmed that the viral message is detected throughout the developing limb with the RCAS system (Fig. 4 *O* and *P*, $n = 30/35$). Consistent with this effect on chondrogenic gene expression, we observed small spot-like ectopic cartilage elements associated with endogenous digits with low efficiency (Fig. 4 *S* and *T*, $n = 5/40$, indicated by red asterisks). These results suggest that *Sox9* alone can promote chondrogenesis in chick limb buds. However, the effect was not significant and was restricted to small regions such as the distal ridge, whereas the transgene was widely expressed.

Correlating with our analysis in MSC, we observed significant effects by cooverexpressing *Sox9* and *PGC-1*α in the developing limb. Double overexpression resulted in ectopic chondrogenesis, shown by ectopic expression of *Col2a1* and *aggrecan*, which is morphologically similar to their expression in digit primordia (Fig. 4 *I–L*, $n = 18/82$ and $n = 3/12$ for *Col2a1* and aggrecan, respectively). In particular, Fig. 4*J* shows a developing leg expressing *Col2a1* in five-digit primordia, whereas the contralateral, normal chick leg contains four-digit primordia (Fig. 4*I*). Also, Fig. 4*L* shows *aggrecan* expression associated with six-digit primordia compared with four in the contralateral leg. In relation with the ectopic expression of these genes, we observed ectopic cartilage similar to endogenous digits (Fig. 4 *U* and *V*, $n = 17/54$, indicated by red arrows). Our results demonstrate that *Sox9* and *PGC-1*α cooperate during chondrogenesis *in vivo* and further support our model that PGC-1α interacts with Sox9 to promote cartilage-specific gene expression and, hence, chondrogenesis.

Discussion

The precise growth and patterning of the developing skeletal framework have been shown to be regulated by the sequential expression of transcription factors (26). Our findings provide evidence that, in addition to these transcription factors, a transcriptional coactivator, PGC-1α, also plays a role in chondrogenesis.

Some coactivators have been shown to be involved in tissue development and differentiation. In view of the fact that many cofactors are widely expressed, PGC-1α is a unique coactivator, because its expression level is dramatically changed during adaptive thermogenesis (27) and muscle fiber type determination (28). Our data further show that *PGC-1*α exhibits dynamic expression during chondrogenesis in the developing limb and differentiating MSC, correlating with the initial process of chondrogenesis (Fig. 1). The *PGC-1*α expression pattern suggests that not only does *PGC-1*α have a specific interaction with

the transcription factor Sox9, but also, that its specific expression might contribute to normal chondrogenesis. This hypothesis is further enhanced by the fact that TGF-β stimulates *PGC-1*α expression during MSC chondrogenesis. Microarray analyses of MSC undergoing chondrogenesis with TGF-β stimulation characterized a number of genes involved in chondrogenesis (5). Although *PGC-1*α was not identified in the analyses, our data clearly demonstrate up-regulation of PGC-1α during chondrogenesis of MSC (Fig. 1*P*). The up-regulation was observed 2 h poststimulation, and this rapid up-regulation suggests that PGC-1α is one of the mediators of TGF-β stimulation during the early process of chondrogenesis. Although it is not known how *PGC-1*α expression is regulated during chondrogenesis, it is noteworthy that *PGC-1*α expression is regulated by cAMP response element-binding (CREB) protein binding protein activity during gluconeogenesis (20, 21). It would be interesting to examine TGF-β-downstream molecule(s) that might regulate *PGC-1*α expression.

The biochemical analyses identified a unique domain of PGC-1α, lying in its amino-terminal region, for direct interaction with Sox9 to activate Sox9-dependent *Col2a1* expression during chondrogenesis (Fig. 2). This finding is consistent with the idea that Sox9 requires a molecular partner(s) for tissue-specific gene regulation and chondrogenesis, as Sox9 also exists in undifferentiated MSC (Fig. 1*Q* and ref. 5) and noncartilage tissues (6). Accumulating evidence has revealed that transcription factors exert their activities through docking with chromatin-modifying factors, as well as coregulatory factors (29). Because Sox9 is also shown to interact with cAMP response element-binding protein binding protein (CBP)/p300 to activate *Col2a1* enhancer (8), it is conceivable that both PGC-1α and CBP/p300 might be part of a larger transcriptional machinery including Sox9.

Our functional analyses with MSC differentiation and developing chick limbs show that PGC-1α functions with Sox9 to regulate chondrogenesis. Beside *Col2a1*, a direct target of Sox9 (23, 24), cooverexpression of *Sox9* and *PGC-1*α activated other chondrogenic gene expressions including those encoding link protein, *COMP*, and aggrecan (Figs. 3 and 4). It is not clear whether these genes are direct targets of Sox9 during chondrogenesis. Nevertheless, these genes are known to be up-regulated during chondrogenesis both in the differentiating MSC and the developing limb buds (4, 5). Therefore, our gene expression analyses suggest that not only was *Col2a1* gene expression initiated by Sox9 and PGC-1α, but the process of chondrogenesis was as well. In agreement with this finding, coexpression of both factors resulted in a significant increase of cartilage matrix accumulation in MSC and in the formation of ectopic digits in the developing chick limb (Figs. 3 and 4). These data further support our theory that PGC-1α regulates chondrogenesis as a coactivator for Sox9.

Our loss-of-function study revealed that the inhibitory effect of *PGC-1*α knockdown on *Col2a1* expression appears to be significant. *PGC-1*α siRNA partially down-regulated *Col2a1* gene expression, whereas PGC-1α protein was almost undetectable (Fig. 3 *A* and *B*). One possible reason is that PGC-1α shares its biological functions with some other factors, such as *PGC-1*β, as shown in other systems (30).

Many coactivators have the potential to interact with multiple transcription factors and regulate their activities. Thus, in addition to Sox9, PGC-1α may regulate other chondrogenesis-related transcription factors during mesenchymal differentiation. Overall, our findings provide insights for having a better understanding of the molecular mechanisms underlying chondrogenesis. Furthermore, regulating chondrogenesis by means of Sox9 and PGC-1α in human MSC opens the possibility for exploring molecular engineering of chondrogenesis in clinical settings.

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